The Plant Cell, Vol. 10, 1193–1206, July 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

Processing of a Composite Large Subunit rRNA: Studies with Chlamydomonas Mutants Deficient in Maturation of the 23S-like rRNA

Stephen P. Holloway and David L. Herrin[1]

Department of Botany and Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas 78713

Maturation of the chloroplast 23S-like rRNA involves the removal of internal transcribed spacers (ITSs) and, in the case of *Chlamydomonas reinhardtii*, the splicing of a group I intron (*Cr.LSU*). Little is known of the *cis* and *trans* requirements or of the processing pathway for this essential RNA. Previous work showed that the ribosome-deficient *ac20* mutant overaccumulates an unspliced large subunit (LSU) RNA, suggesting that it might be a splicing mutant. To elucidate the molecular basis of the *ac20* phenotype, a detailed analysis of the *rrn* transcripts in *ac20* and wild-type cells was performed. The results indicate that processing of the ITSs, particularly ITS-1, is inefficient in *ac20* and that ITS processing occurs after splicing. Deletion of the *Cr.LSU* intron from *ac20* also did not alleviate the mutant phenotype. Thus, the primary defect in *ac20* is not splicing but most likely is associated with ITS processing. A splicing deficiency was studied by transforming wild-type cells with *rrnL* genes containing point mutations in the intron core. Heteroplasmic transformants were obtained in most cases, except for P4 helix mutants; these strains grew slowly, were light sensitive, and had an RNA profile indicative of inefficient splicing. Transcript analysis in the P4 mutants also indicated that ITS processing can occur on an unspliced precursor, although with reduced efficiency. These latter results indicate that although there is not an absolutely required order for LSU processing, there does seem to be a preferred order that results in efficient processing in vivo.

INTRODUCTION

The rRNA genes (*rrn*) of chloroplasts resemble their prokaryotic ancestors not only in that they have similar primary coding sequences but also in that they typically exist in an operon. The order of the major *rrn* genes in the choroplast is similar to eubacteria: these are 16S, 23S, and 5S, respectively (reviewed in Harris et al., 1994). In addition, isoleucine and alanine tRNA genes typically are encoded in the spacer between 16S and 23S, and other tRNA genes often flank the *rrn* operon (Harris et al., 1994). Thus, the primary transcript must undergo considerable processing to generate the RNAs found in plastid ribosomes. This process is poorly understood in chloroplasts as compared with *Escherichia coli*, which has been well studied (reviewed in Gegenheimer and Apirion, 1981). One approach that has been used in *E. coli* is the isolation of mutants defective in pre-rRNA processing. These mutants were instrumental in providing in vivo data on the enzymes and intermediates in the processing pathway (Gegenheimer and Apirion, 1981).

A distinctive feature of chloroplast rRNA processing concerns the 23S-like large subunit (LSU) rRNA. When RNA from chloroplast ribosomes is separated under nondenaturing conditions, a major band that comigrates with *E. coli* 23S rRNA typically is seen. However, when the same RNA is separated under denaturing conditions, this band is absent or greatly reduced and smaller RNAs appear (e.g., Leaver and Ingle, 1971).

In angiosperms, a 4.5S RNA is associated with the LSU, which is similar in sequence to the 3′ terminus of *E. coli* 23S. The DNA sequence for 4.5S rRNA, however, is separated from upstream 23S sequences by a short sequence that shows no similarity to *E. coli* 23S (reviewed in Harris et al., 1994). The rest of the 23S-like RNA also is not continuous, being fragmented in two places. Preliminary data indicate that these interruptions may be very small (i.e., several nucleotide) gaps (Kössel et al., 1985).

In the green alga *Chlamydomonas reinhardtii*, the 4.5S rRNA is absent, but the ribosomal LSU contains two other small RNAs, 7S and 3S, which are similar in sequence to the 5′ end of *E. coli* 23S. These RNAs are also encoded at the 5′ end of the chloroplast 23S gene but are separated from each other and the remainder of 23S by sequences that are dissimilar to any 23S sequences (Rochaix and Malnoe, 1978; Rochaix and Darlix, 1982). Recently, the Chlamydomonas 23S gene was shown to contain a third internal gap or spacer that fragments the remaining portion of 23S

[1]To whom correspondence should be addressed. E-mail bohl734@utxvms.cc.utexas.edu; fax 512-471-3878.

rRNA into 821- and 1696-nucleotide RNAs, respectively (Turmel et al., 1993). This spacer, like the others in 23S-like *rrn* genes, is not conserved in sequence or structure. Turmel et al. (1993) have argued that the absence of these sequences from the mature RNA are due to specific in vivo processing events. A similar suggestion was made for the fragmented 23S rRNA in land plant chloroplasts (Kössel et al., 1985). Almost nothing is known, however, about these putative processing events. In fact, in vivo evidence for these being bona fide processing sites is lacking in *Chlamydomonas* spp, although there is such evidence for angiosperms (Hartley, 1979; Keus et al., 1984).

In addition to internal transcribed spacers (ITSs), some chloroplast 23S-like *rrn* genes also contain one or more introns (Turmel et al., 1993; Kapoor et al., 1997). The Chlamydomonas gene contains a single group I intron (*Cr.LSU* in the nomenclature for group I introns; Dujon, 1989), which is capable of self-splicing in vitro (Herrin et al., 1990). This intron also undergoes intron homing, which is a recombinatory process that results in the intron invading intronless copies of the 23S gene. The homing of *Cr.LSU* is promoted by the I-CreI endonuclease, which is encoded by the intron's open reading frame (ORF; Durrenberger and Rochaix, 1991; Thompson et al., 1992; Durrenberger et al., 1996).

Although *E. coli* is a valuable system for studying rRNA maturation in prokaryotes, it offers limited insights into these secondary processing events occurring in chloroplasts. In this sense, Chlamydomonas offers a unique opportunity to elucidate chloroplast rRNA maturation, because both classic and reverse genetic approaches are possible. Several mutants affected in chloroplast ribosome biogenesis have been isolated. Most of them are nuclear derived; however, at least two chloroplast loci are implicated (Shepherd et al., 1979; Harris et al., 1987). These mutants are typically leaky, at least at normal growth temperatures, presumably because stringent mutants in chloroplast protein synthesis would not be viable. Although the precise natures of the nuclear mutations have yet to be determined, characterization of these strains has provided insight into the complex molecular interactions associated with biogenesis of chloroplast ribosomes (Myers et al., 1984; Schmidt et al., 1985; Liu et al., 1988).

One of the first chloroplast ribosome–defective mutants identified in Chlamydomonas is *ac20*, which was isolated based on its requirement for acetate for vigorous growth (Boynton et al., 1970). Sedimentation analysis indicated that there are reduced levels of intact chloroplast ribosomes in *ac20* and increased levels of the ribosomal subunits compared with wild-type cells (Boynton et al., 1970; Harris et al., 1974). When grown mixotrophically (i.e., light plus acetate), *ac20* has a doubling time that is 35% greater than that of the wild type. Recently, it was suggested that the defect in *ac20* might lie in splicing of the *Cr.LSU* intron. This was based on the overaccumulation of an unspliced form of 23S pre-RNA (Herrin et al., 1990); however, the possibility of other problems with rRNA processing was not ruled out.

We have sought to resolve the molecular phenotype of *ac20* by (1) performing a comprehensive analysis of rRNA transcripts (in *ac20* and in wild-type cells) and (2) deleting the *Cr.LSU* intron, with the expectation that this should restore a wild-type phenotype to *ac20* if it is in fact an rRNA splicing mutant. This latter approach was inspired by experiments with yeast in which splicing-deficient mutants were restored to a wild-type phenotype by intron deletion (e.g., Hill et al., 1985; Séraphin et al., 1987), although in those cases it was accomplished by standard genetic means rather than organelle transformation. Finally, we have examined the consequences of *cis*-acting mutations that reduce *Cr.LSU* splicing. This was accomplished by altering specific regions of the intron's core.

RESULTS

RNA Gel Blot Analysis of Transcripts from the *rrn* Region

RNA gel blot hybridization was used to analyze the size, composition, and relative abundance of transcripts from the chloroplast *rrn* operon in *ac20* and wild-type cells. Figure 1 shows the locations of the probes that were used. An alternative nomenclature exists for the 23S-like RNAs that are internally processed (Turmel et al., 1991); the whole RNA is called LSU (for large subunit of the ribosome) rRNA, and the processed products, from the 5′ end, are α, β, γ, and δ, which correspond to the 7S, 3S, and the small and large fragments of 23S, respectively, in an earlier nomenclature (Rochaix and Darlix, 1982). We have retained the 7S, 3S, and 23S labels, because these are well established. However, we refer to the whole, composite RNA as LSU rRNA and use 23Sγ and 23Sδ to refer to the RNAs derived by internal processing of the rRNA previously labeled as 23S (Rochaix and Darlix, 1982).

RNA gel blot analysis with the 16S, 5S, and tRNA-containing spacer probes revealed little or no difference between *ac20* and wild-type cells. In addition, there was no detectable accumulation of precursors containing 16S or 5S rRNA sequences (data not shown). However, as shown in Figure 2, the LSU probes detected precursor RNAs in both strains, and significant differences were observed between *ac20* and the wild type. The RNA gel blots for each strain are presented separately to facilitate comparisons with different probes; however, RNA from each strain was also blotted and hybridized together to each probe (data not shown). To facilitate interpretation of the blots, representative diagrams of each are presented in Figure 2, and the major precursor transcripts are drawn to the right. Finally, the exact sizes of the various components of the *rrnL* gene are given in Table 1.

As shown in Figure 2A, there was almost complete conversion of LSU pre-RNA to the final products in wild-type cells, with $<$2% of the hybridizing RNA in precursor form. The largest precursor observed corresponded to an essentially

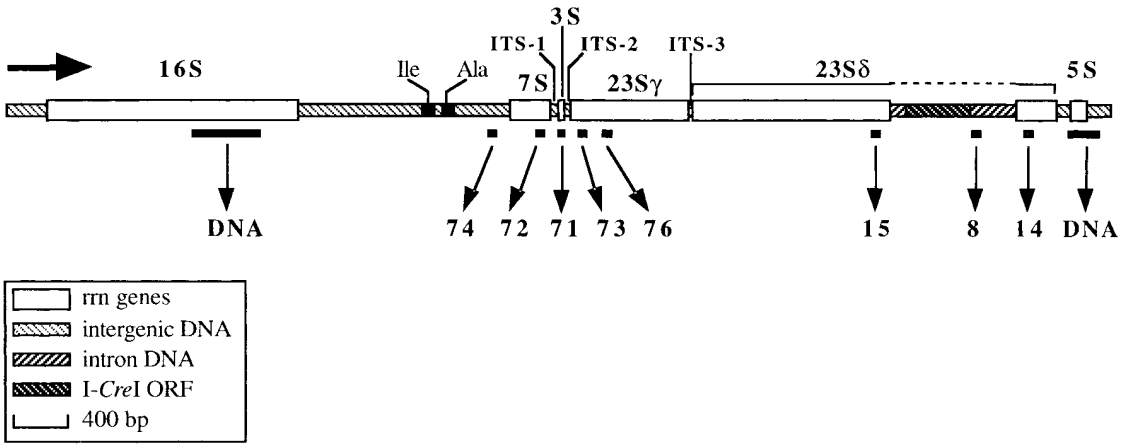

Figure 1. Map of the Chloroplast *rrn* Region and the Locations of Oligodeoxynucleotide and Cloned DNA Probes Used in This Study.

The oligodeoxynucleotide probes, which are indicated in the diagram numerically (see also Methods), were synthesized to hybridize with the sense strand, with the exception of oligodeoxynucleotide 15, which was used for polymerase chain reaction (PCR). Restriction fragments of plasmid DNA (DNA) were used to probe for 16S (EcoRI fragment of pES7.2; Thompson and Herrin, 1991) and 5S (HindIII-BamHI fragment of pSP6523S.4; Herrin et al., 1990) transcripts, respectively. The horizontal arrow indicates the direction of transcription. The structure and sequence of the *rrn* operon are from Rochaix and Malnoe (1978), Dron et al. (1982), Rochaix and Darlix (1982), Rochaix et al. (1985), Schneider et al. (1985), Schneider and Rochaix (1986), and Lemieux et al. (1989). See the text for a discussion of the nomenclature used for the 23S coding regions. Ile and Ala, isoleucine and alanine *trn* genes.

unprocessed LSU pre-RNA, hereafter called 7S-3S-23Sγ-23Sδ-intron RNA (3803 nucleotides in Figure 2). The level of this RNA was relatively low, and long-exposure times were required to visualize it in wild-type RNA; those exposures also resulted in drastic overexposure of the mature RNA bands when the coding-region probes were employed. Thus, in Figure 2A, this precursor is only visible in the lane hybridized with the intron probe (in Figure 2B, however, it is visible in all lanes); the 889-nucleotide RNA that hybridizes with the intron probe is the excised, linear intron (Herrin et al., 1990). The next largest precursor corresponds to the aforementioned RNA without the intron, that is, 7S-3S-23Sγ-23Sδ (2915 nucleotides in Figure 2), whereas the third and final precursor detected in wild-type cells corresponds to a 23Sγ-23Sδ species (2528 nucleotides in Figure 2). The composition of these precursors suggests that, in wild-type cells, splicing precedes ITS processing and that processing of ITS-1 and ITS-2 precedes removal of ITS-3.

The two 23Sγ probes (see Figure 1) unexpectedly revealed two strongly hybridizing bands in wild-type RNA. Each probe detected a band close to the expected size of 23Sγ (821 nucleotides) and also a smaller RNA, which was different for each probe (indicated with stars in the diagrams shown in Figure 2). The sizes of the smaller RNAs, ~140 nucleotides and ~680 nucleotides, respectively, taken together with the locations of the two probes suggest that there exists a hitherto unknown processing or cleavage site within 23Sγ, ~140 nucleotides from the 5′ end. The data also indicate that only ~35% of the cellular 23Sγ RNA is cleaved at this site. This latter point was confirmed, and the site was mapped by primer extension (see below and Figure 3).

In marked contrast to wild-type cells, Figure 2B shows that a considerable fraction (at least 30%) of the LSU rRNA in *ac20* is in precursor form. Beginning with the largest observed precursor, there is a considerable increase in the 7S-3S-23Sγ-23Sδ-intron RNA (3803 nucleotides) in *ac20* compared with wild-type cells. This precursor is most certainly the same as that detected previously with an intron-specific probe and shown to be capable of self-splicing in vitro (Herrin et al., 1990). Despite the substantial increase in this RNA in the mutant, this transcript remains at a very low level, as evidenced by its relatively weak signal with the coding-region probes (Figure 2B, lanes 1 to 4 and 6) and the extended exposure times required with the intron probe (Figure 2B, lane 5, and legend). The next largest precursor, which is also the most abundant in *ac20*, corresponds to the 7S-3S-23Sγ-23Sδ species (2915 nucleotides) seen in wild-type cells (Figure 2B). A 23Sγ-23Sδ RNA (2528 nucleotides) also appears to be present, although this species is difficult to distinguish from a 3S-23Sγ-23Sδ RNA (2600 nucleotides), because of the very strong signal from the 7S-3S-23Sγ-23Sδ transcript (2915 nucleotides) in *ac20.*

The RNA gel blot analysis of *ac20* also revealed two novel RNAs not observed in wild-type cells, a 7S-3S-23Sγ species (1208 nucleotides) and a 7S-3S species (362 nucleotides) (Figure 2B). The accumulation of these two RNAs plus the drastic overaccumulation of the 7S-3S-23Sγ-23Sδ precursor indicate that processing of the ITSs, particularly ITS-1, is

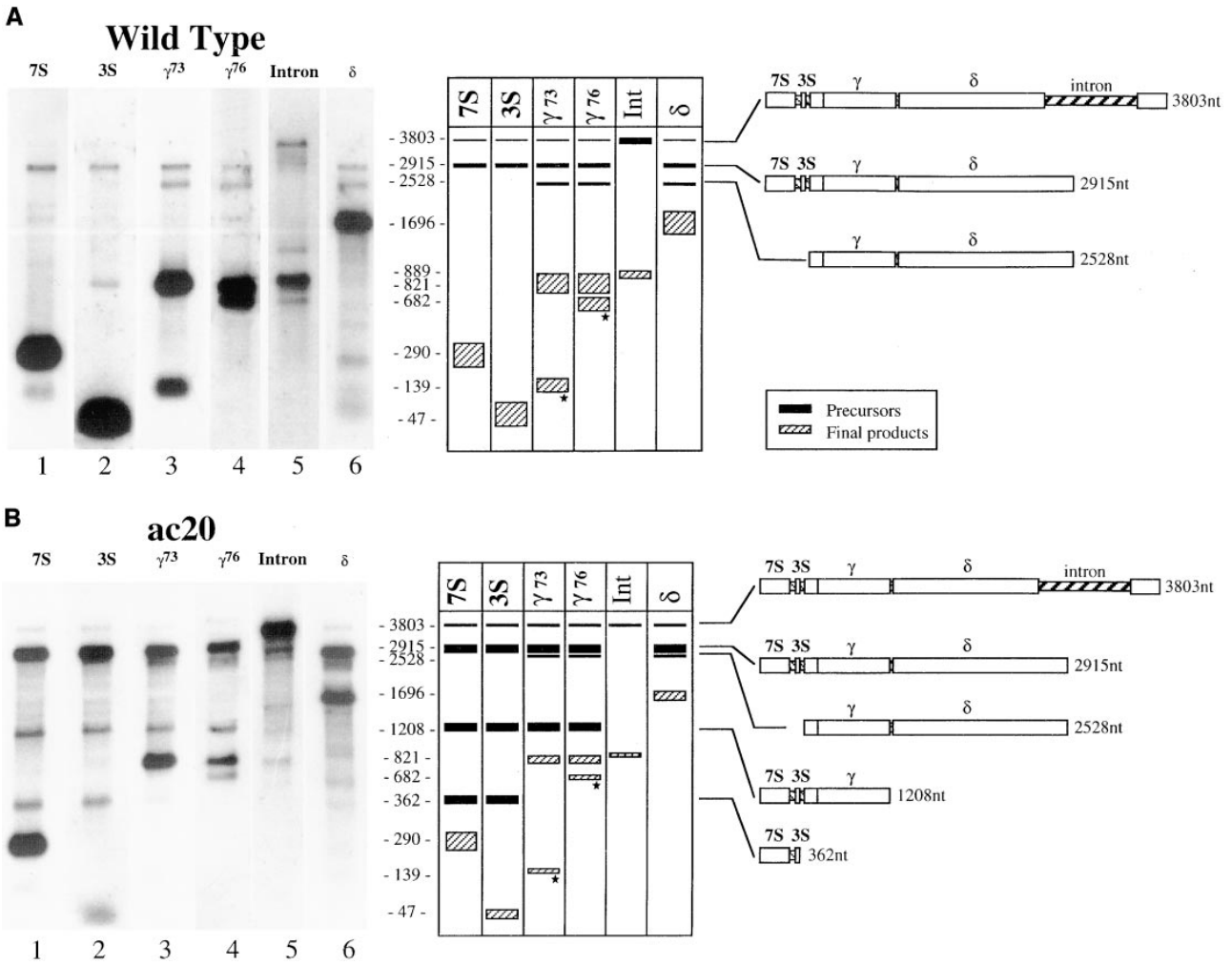

Figure 2. RNA Gel Blot Analysis of Wild-Type and *ac20* RNA with LSU (23S) Probes.

Blots of total RNA (2.5 μg per lane) were hybridized with probes specific for the component indicated above each lane (see also Figure 1). The exact exposure times varied, depending on the specific activity of the probe; however, in general, the blots hybridized with the intron-specific probe were exposed to film three or four times longer than were the others. The sizes of the RNAs, which are indicated to the right of the gels (in nucleotides), are based on published values (see Table 1); they are also consistent with RNA size markers (Boehringer Mannheim) that were visible on the stained blots.

(A) Blots of RNA from wild-type cells (Wild Type).

(B) Blots of RNA from the *ac20* mutant (ac20).

To the right in **(A)** and **(B)** is a diagram of the LSU rRNAs observed with each probe; only the major transcripts that were observed with several different RNA preparations are indicated. The sizes of the boxes that represent the RNA bands are approximately proportional to the hybridization signals. The two boxes in each diagram that are underscored with a small star are the products of the partial cleavage of 23Sγ. The vertical line near the 5′ end of 23Sγ (compare with Figure 1) indicates the cleavage site. The structures and sizes of the precursor RNAs are indicated to the right of each diagram. Int, intron; nt, nucleotides.

substantially impaired in *ac20.* These results also indicate that processing of ITS-2 and ITS-3 can occur in the absence of ITS-1 processing. Finally, it should be noted that there also appears to be a significant reduction in the internal cleavage of 23Sγ in *ac20* (see Figure 2B, lane 4). The smaller (~140 nucleotides) product of 23Sγ cleavage was only visible (lane 3) with a long exposure (not shown).

Primer Extension Analysis of 23Sγ Cleavage

Because internal cleavage of 23Sγ had not been reported previously, we mapped the cleavage site by using primer extension with reverse transcriptase. As shown in Figure 3A, primer extension of wild-type RNA with oligodeoxynucleotide 76 gave two major cDNA products: the smaller of

these is 135 nucleotides in length and represents the 5′ end of the large (~680 nucleotides) 23Sγ subfragment, whereas the larger extension product was sized at 270 ± 5 nucleotides, which is in the range expected for the 5′ end of 23Sγ (Turmel et al., 1993). When oligonucleotide 73, which as it turns out overlaps by two bases into the large 23Sγ subfragment (Figure 3B), was used for primer extension, a single band of 142 nucleotides was obtained (data not shown), which is in perfect agreement with the 5′ terminus of 23Sγ determined by Turmel et al. (1993). Figure 3C shows the location of the 5′ terminus of the large 23Sγ subfragment. The indicated nucleotide corresponds to A505 in *E. coli* 23S rRNA, which lies in a region (domain I) that is highly conserved, from prokaryotes (including *E. coli*) to higher plant chloroplasts (Gutell et al., 1990).

The internal cleavage of 23Sγ does not appear to be the removal of a spacer, because oligonucleotide 73 (Figure 3B) hybridized strongly with the small upstream fragment of 23Sγ on RNA gel blots (Figure 2). This would not have been possible if more than one or two nucleotides had been removed from the 3′ terminus of this RNA, although we cannot rule out the possibility that a very small (i.e., one to two nucleotides) gap was introduced into the primary sequence. This seems very unlikely, however, given the strong conservation of nucleotides on both sides of this site (Gutell et al., 1990).

Deletion of the *Cr.LSU* Intron from *ac20* and the Wild Type

It was suggested previously that the *ac20* phenotype might result from a deficiency in splicing of the *Cr.LSU* intron (Herrin et al., 1990). This hypothesis was tested by deleting the *Cr.LSU* intron from *ac20*, with the expectation that intron deletion would restore a wild-type phenotype to *ac20* if its primary problem was inefficient splicing. We also decided to delete the intron from wild-type cells to assess any potential effects in a wild-type background. Gene replacement is possible in this system, because chloroplast transformation proceeds via homologous recombination (e.g., Newman et al., 1990). However, because *Cr.LSU* is capable of intron homing (Durrenberger et al., 1996), the task had to be performed in two sequential steps. First, the recipient strains (*ac20* and wild type) were transformed to ORF-minus (*ORF*$^-$) by using the plasmid pES7.2 (Thompson and Herrin, 1991), which is similar to the construct shown in Figure 4A. These transformants thus lacked the ability to convert intron-minus (*int*$^-$) DNA to intron-plus via intron homing (Durrenberger et al., 1996). They were selected by plating on spectinomycin, and then the primary transformants were tested for erythromycin resistance. Homoplasmicity of the doubly resistant transformants was checked by the polymerase chain reaction (PCR) with oligodeoxynucleotides 14 and 15 (see Figure 1) and confirmed by DNA gel blot hybridization with an intron-specific probe (data not shown).

Table 1. The Composite *rrnL* Gene of Chlamydomonas

Component	Length[a]
7S rRNA	290
ITS-1	25
3S rRNA	47
ITS-2	25
23Sγ rRNA	821
ITS-3	11
23Sδ rRNA	1696
Cr.LSU intron	888

[a] The lengths are in nucleotides and are taken from Rochaix et al. (1985) and Turmel et al. (1993).

Homoplasmic *ORF*$^-$ transformants of *ac20* and the wild type were then used in a second transformation to *int*$^-$. This utilized the plasmid pGEM23S.int$^-$ shown in Figure 4B, which, in addition to lacking the intron, carries streptomycin resistance in the 16S gene and 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) resistance in a nearby exon (exon 5) of the *psbA* gene (Erickson et al., 1984). Transformation with pGEM23S.int$^-$ also removed the spectinomycin and erythromycin markers from the previous transformation. The initial selection of transformants was with streptomycin, secondary selection was on DCMU, and homoplasmicity of the transformants was checked by PCR (Figure 5A) and DNA gel blot hybridization (data not shown). A number of homoplasmic *int*$^-$ transformants were obtained in the wild-type and *ac20* genetic backgrounds, and Figure 5A shows the results of PCR analysis of a representative transformant of each strain. Figure 5A also shows the PCR analysis of an *ac20* transformant after the first transformation to *ORF*$^-$. In each of the PCR reactions, only a single DNA product of the correct size was obtained, indicative of a homoplasmic chloroplast genome.

The effect of intron deletion on growth and cell morphology was determined in Tris–acetate–phosphate (TAP) liquid culture; neither the wild-type nor *ac20* transformants showed any observable alteration of either cell morphology or growth rate compared with its recipient intron-plus. The same was true on solid TAP medium, with the exception that *ac20 int*$^-$ grew just perceptibly faster than did *ac20.* However, there was no amelioration of the cold sensitivity (on solid media) associated with the *ac20* mutation (data not shown). RNA gel blot analysis of two *ac20 int*$^-$ clones with 23Sδ and intron probes is shown in Figures 5B and 5C, respectively. The unspliced 7S-3S-23Sγ-23Sδ-intron RNA (3803 nucleotides) is absent from the *int*$^-$ transformants (Figure 5B), as expected. Figure 5B also shows that processing of the 7S-3S-23Sγ-23Sδ RNA (2915 nucleotides) is still inefficient in the *ac20 int*$^-$ strains. These results indicate that the reduction in *Cr.LSU* splicing efficiency in *ac20* is not primarily responsible for the *ac20* phenotype. They also demonstrate that the *Cr.LSU* intron does not encode anything essential for cell growth.

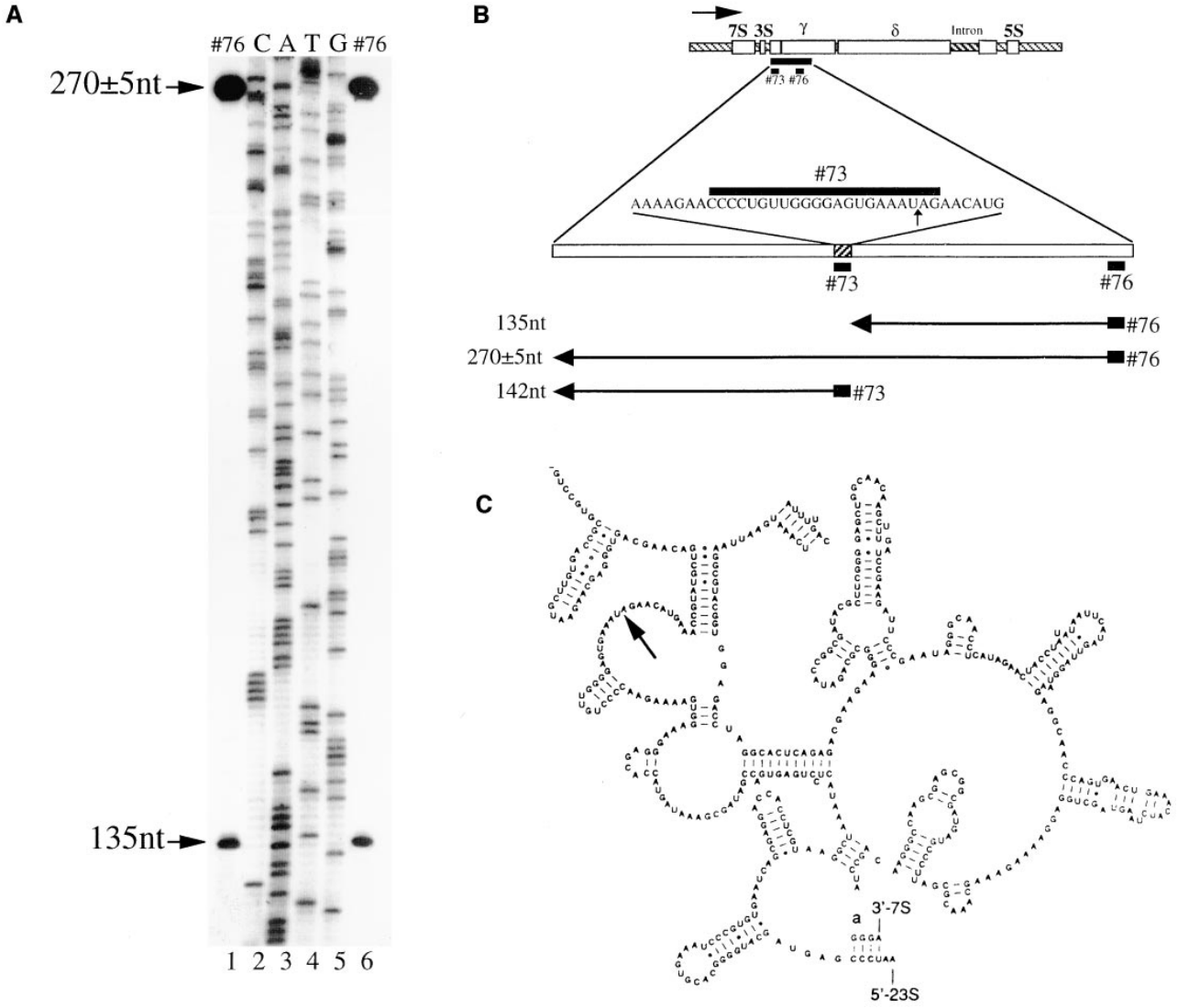

Figure 3. Primer Extension Analysis of the Internal Cleavage of 23Sγ.

An end-labeled primer, either oligodeoxynucleotide 76 or 73 (data not shown), was annealed to total cellular RNA from wild-type cells and then extended with reverse transcriptase. The extension products were separated by electrophoresis on a 6% polyacrylamide/8 M urea gel alongside a DNA sequence ladder generated with the same primer.

(A) Primer extension with oligodeoxynucleotide 76. The sizes of the major extension products are indicated to the left of the autoradiograph in nucleotides (nt). Lanes 1 and 6 contain duplicate reactions.

(B) Diagram showing the location of the primers and the corresponding extension products (and their sizes). The horizontal arrows indicate the direction of reverse transcription and the map positions of the major extension products. The small vertical arrow in the expanded sequence region marks the 5′ terminus of the large subfragment of 23Sγ.

(C) Structure of the region of 23S rRNA of Chlamydomonas (from Gutell et al., 1990) that includes domain I; the cleavage site in 23Sγ determined by primer extension is indicated by an arrow.

Transformation of Wild-Type Cells with *Cr.LSU* Core Mutants

To study the effects of a splicing deficiency in vivo, we used site-directed mutagenesis to introduce point mutations in the P4, J4/5, P6, and P7 regions of the *Cr.LSU* intron in vitro. Domains P4, P6, and P7 are paired regions that are part of the conserved core and considered to be essential for group I ribozyme activity (e.g., Michel and Westhof, 1990); the induced mutations were expected to destabilize

these helices (see Methods). J4/5 is an unpaired region that joins helices P4 and P5 and contains a conserved A residue that is believed to be important for docking of the 5′ splice site domain with the catalytic core (e.g., Michel and Westhof, 1990); the conserved A was changed to a C. A *rrnL* gene with an *ORF*⁻ intron was used for the mutagenesis and subsequent gene replacement to allow the use of PCR to assess homoplasmicity. A mutation conferring spectinomycin resistance was also incorporated into the plasmids (see Figure 4A) before their transformation into wild-type Chlamydomonas. It should be noted that these latter two markers have no affect on *Cr.LSU* splicing in vivo or in vitro (Thompson and Herrin, 1991).

Only transformants with a point mutation in the P4 helix could be obtained in homoplasmic form. The other intron mutations were apparently too severe to permit gene replacement. Figure 6A shows the PCR analysis of two P4 mutants, *SH19* (lane 2), which contains a C-126–to–A substitution,

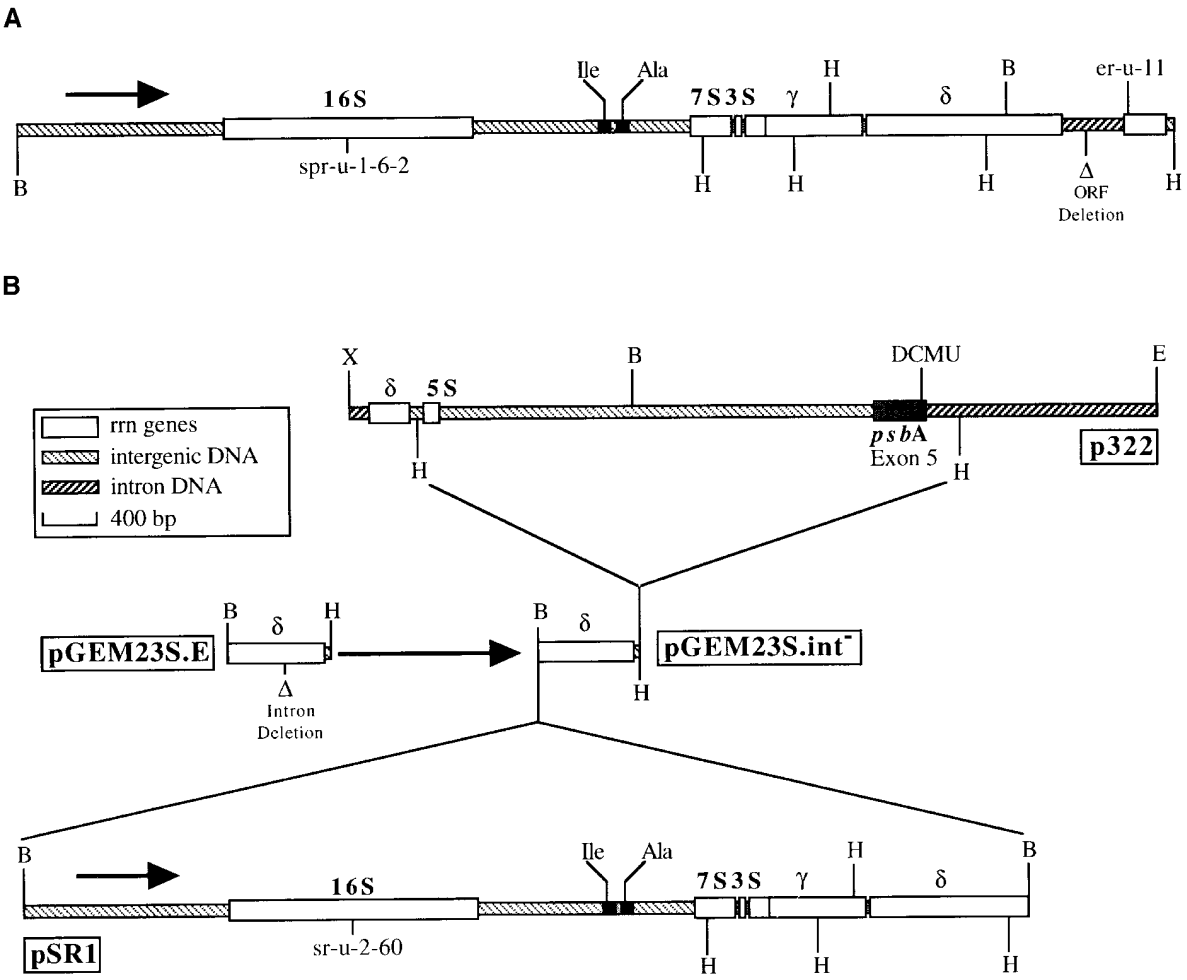

Figure 4. Chloroplast Transformation Plasmids.

(A) Map of the plasmids used to replace the wild-type *Cr.LSU* intron with versions containing single-point mutations in the intron core. Labeling of the *rrn* coding regions is as given in Figure 1.

(B) Map of the pGEM23S.int⁻ plasmid used to delete the *Cr.LSU* intron. A complete *rrn* operon was reconstructed around an intron-deleted BamHI-HindIII fragment of the 23S gene from pGEM23S.E (Thompson et al., 1992). The 3′ sequences were added from the plasmid p322 (Newman et al., 1992) as a HindIII fragment, which also contained exon 5 of a DCMU-resistant *psbA* gene (Erickson et al., 1984). Subsequently, the 5′ sequences were added as a BamHI fragment from the plasmid pSR1, whose 16S gene contains a streptomycin resistance marker (Newman et al., 1990).

B, BamHI; E, EcoRI; H, HindIII; X, XhoI; Δ ORF Deletion, ORF⁻; Δ Intron Deletion, int⁻; Ala and Ile, alanine and isoleucine *trn* genes; er-u-11, erythromycin resistance marker; spr-u-1-6-2, spectinomycin resistance marker; sr-u-2-60, streptomycin resistance marker; DCMU, DCMU resistance marker.

and *SH34*, which contains a C-126–to–G substitution; only one band of the expected size was obtained in each PCR reaction, indicative of homoplasmicity. DNA gel blot analysis also confirmed that the wild-type intron was not present in these strains (data not shown). Finally, the PCR products of Figure 6A were sequenced using oligodeoxynucleotide 8 to verify the presence of the mutations (data not shown).

Figure 6B shows the growth of the transformants on solid media compared with wild-type cells. Both types of P4 mutants grew slower than did the wild type and were sensitive to bright light. *SH34* was very sensitive to light and did not grow at all under high light intensity. Both mutant strains also showed partial cold sensitivity (16°C; data not shown).

Despite the apparent differences in growth rates, RNA gel blot analysis gave fairly similar RNA profiles for the P4 mutants; thus, data for only one of these strains, *SH19*, are presented in Figure 6C. Splicing was strongly affected, as was subsequent LSU RNA processing; however, as with the wild type and *ac20*, it was not possible to detect precursors containing 16S, the 16S/7S spacer, or 5S.

The largest precursor in *SH19* corresponds to a 7S-3S-23Sγ-23Sδ-intron species (3437-nucleotide RNA in Figure 6), followed by what appears to be a 23Sγ-23Sδ-intron RNA (3050 nucleotides). However, this band may also harbor a 3S-23Sγ-23Sδ-intron RNA, because the expected size difference (72 nucleotides) compared with that of a 23Sγ-23Sδ-intron RNA is probably not great enough to allow resolution on this gel. The smallest precursor observed was a 23Sδ-intron species (2218-nucleotide RNA in Figure 6), which appeared as a doublet of bands. Why this RNA appears as a doublet is not clear; however, it is not because of the presence or absence of 23Sγ. It may represent heterogeneity at the 3′ terminus of 23Sδ (Y.-F. Chen and D.L. Herrin, unpublished data). Finally, the nature of the ~140-nucleotide RNA that hybridizes to the 7S probe (Figure 6C, lane 1) is not known; however, it is not the 23Sγ subfragment, because the 7S and 23Sγ probes are separated by >250 nucleotides.

In conclusion, these results indicate that it is possible to replace the wild-type *Cr.LSU* intron with a splicing-deficient version as long as it is not completely blocked in splicing; the P4 mutant introns were also capable of self-splicing in vitro but only at higher than normal Mg^{2+} concentrations (data not shown). The accumulation of the partially processed, intron-containing precursors in these strains indicates that ITS processing can occur on unspliced RNA; however, the efficiency seems reduced (based on the considerable accumulation of the precursor containing the intron and all three ITSs).

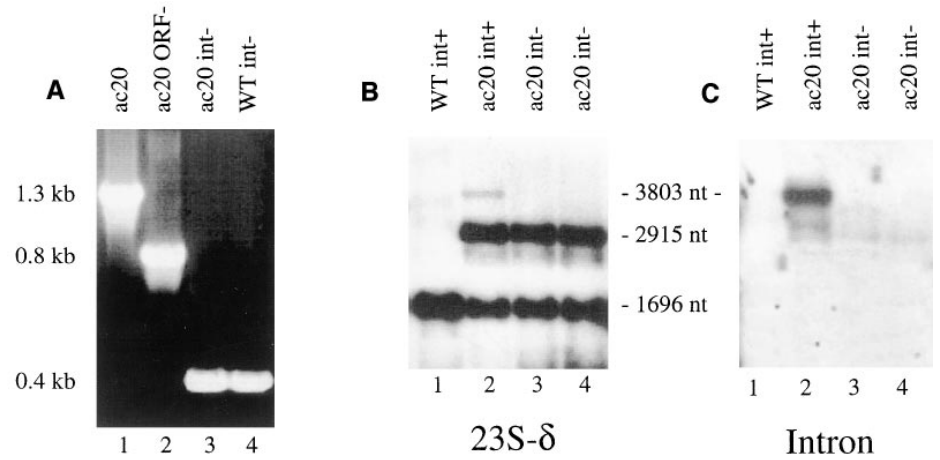

Figure 5. Deletion of the *Cr.LSU* Intron from *ac20* and Wild-Type Chlamydomonas.

Intron deletion was a two-step process. Initially, the ORF was deleted to prevent the intron from homing into intronless DNA. This was accomplished by transformation of wild-type (WT) with plasmid pES7.2 (Thompson and Herrin, 1991). Subsequently, the remainder of the intron was removed by transformation of the ORF$^-$ strain with an intronless *rrnL* gene (see Figure 4B).

(A) PCR test for homoplasmicity of the transformants. PCR products from *ac20* (lane 1), *ac20 ORF*$^-$ (lane 2), *ac20 int*$^-$ (lane 3), and WT *int*$^-$ (lane 4) were analyzed by agarose gel electrophoresis and ethidium bromide staining; the PCR primers flank the intron (oligodeoxynucleotides 14 and 15 in Figure 1). Band lengths are indicated at left in kilobases. The markers were HindIII-digested λ and HaeIII-digested ΦX174 DNAs.

(B) RNA gel blot analysis of *ac20 int*$^-$ transformants. Total RNA (2.5 μg per lane) from the wild type (lane 1), *ac20* (lane 2), and two different *ac20 int*$^-$ transformants (lanes 3 and 4) was hybridized with the 23Sδ probe (oligodeoxynucleotide 14 in Figure 1). The composition of the RNA bands whose lengths, in nucleotides (nt), are given here are as follows: 3803-nucleotide RNA, 7S-3S-23Sγ-23Sδ-intron; 2915-nucleotide RNA, 7S-3S-23Sγ-23Sδ; and the 1696-nucleotide RNA, 23Sδ.

(C) RNA gel blot analysis of the same RNA preparations used for **(B)** but hybridized with the intron-specific probe (oligodeoxynucleotide 8 in Figure 1). The origin of the faint signals migrating faster than the unspliced 7S-3S-23Sγ-23Sδ-intron RNA (3803 nucleotides) is not known.

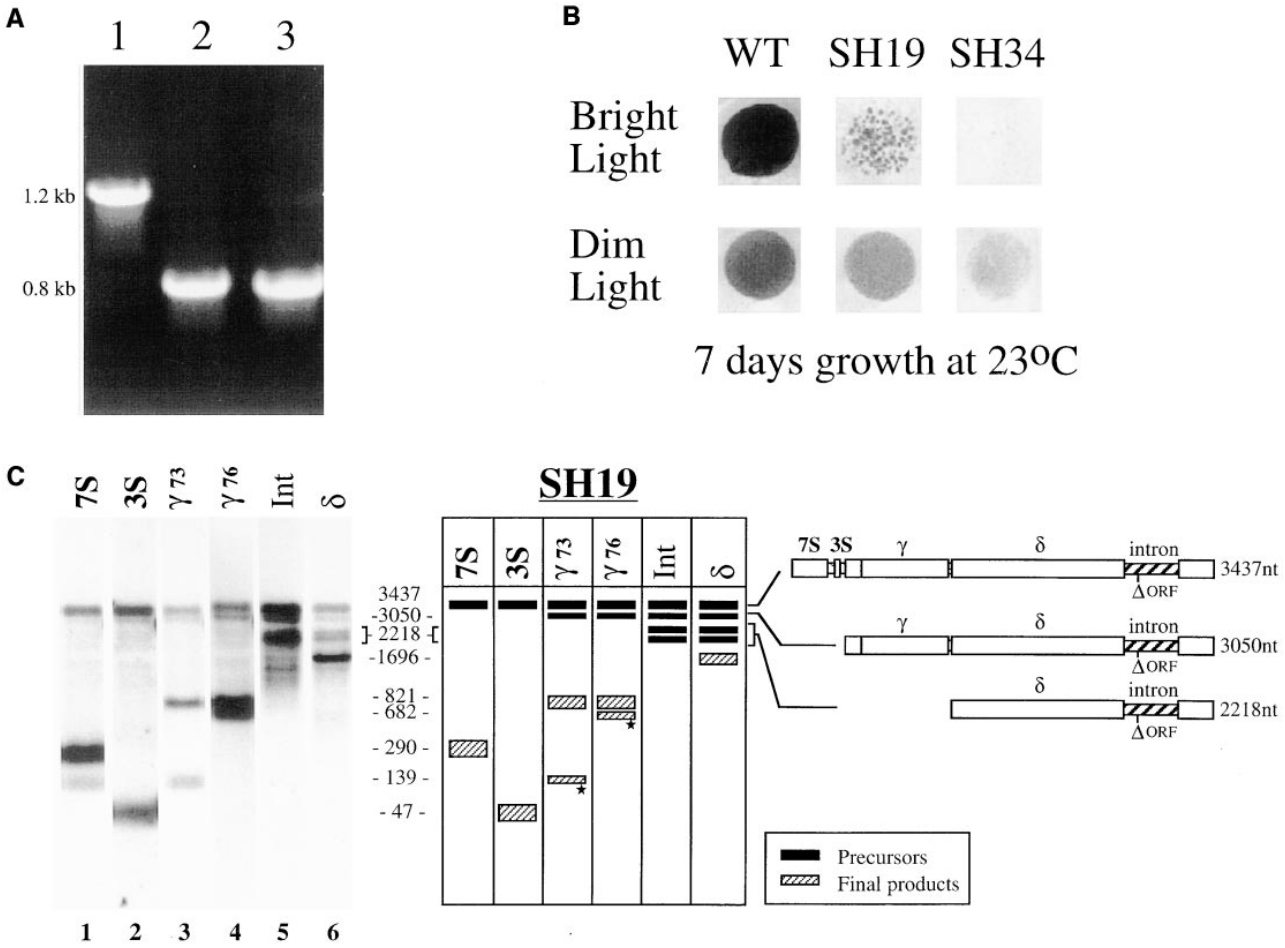

Figure 6. Transformation of Wild-Type Chlamydomonas with an *rrnL* Gene Containing Nucleotide Substitutions in the P4 Helix of the Intron.

Strains *SH19* and *SH34* were created by transformation of wild-type Chlamydomonas with DNA similar to that shown in Figure 4A that also contained a single nucleotide substitution in the P4 helix of the intron. *SH19* has a C-126–to–A change, whereas *SH34* has a C-126–to–G change; both changes are expected to weaken the P4 helix (see Thompson and Herrin [1991] for the structure of P4).

(A) PCR analysis of homoplasmicity. PCR products are from the wild type (lane 1), *SH19* (lane 2), and *SH34* (lane 3) strains; the primers flank the intron (14 and 15 in Figure 1). The lengths of the bands are indicated at left in kilobases. The DNA markers were HindIII-digested λ and HaeIII-digested ΦX174 DNAs.

(B) Comparison of the growth of the *SH19* and *SH34* strains to the wild type (WT) on solid TAP media; bright light was ~5000 lux; dim light was ~150 lux.

(C) RNA gel blot analysis of the P4 mutant *SH19.* Total RNA (2.5 μg per lane) was hybridized with the indicated probes (see also Figure 1). The exposure time was approximately fourfold longer for the intron-specific probe than for the others. The RNA lengths indicated to the right of the blots are in nucleotides and are based on the published sizes of LSU components (Table 1; the ORF-deleted intron in *SH19* is 522 nucleotides [Thompson and Herrin, 1991]); they are also consistent with the RNA size markers (Boehringer Mannheim) that were visible on the stained blot. The observed RNAs are also diagrammed to the right, with the structures of the precursors indicated on the far right. The bands underscored with a small star are the products of the 23Sγ internal cleavage (which is always partial); the cleavage site in 23Sγ is indicated by a vertical line near the 5′ end. nt, nucleotides.

DISCUSSION

rRNA Processing in the *ac20* Mutant

Mutants whose primary defect is in chloroplast LSU rRNA processing have not been reported to our knowledge, although a maize mutant, *hcf7*, which is deficient in chloroplast 16S rRNA processing, has been described (Barkan, 1993). The *ac20* mutant of Chlamydomonas is clearly deficient in processing the chloroplast LSU rRNA, accumulating large amounts of partially processed precursors (Figure 2). The effects of this nuclear mutation are pleiotropic in that multiple steps in LSU processing are affected, albeit not

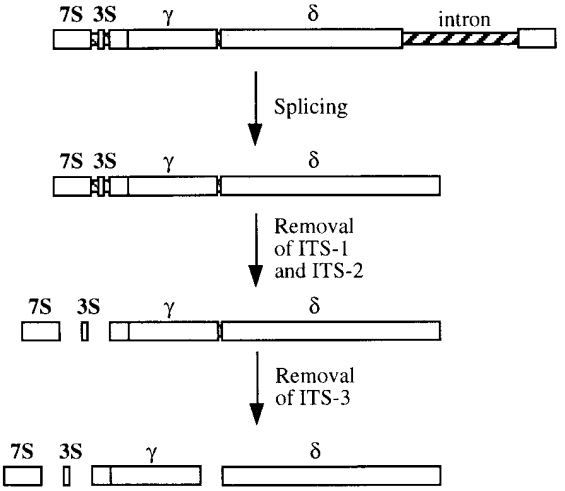

Figure 7. Proposed Pathway for LSU Pre-rRNA Processing in Wild-Type Chlamydomonas.

See Figure 1 for a legend for the *rrnL* gene components, and see the text for discussion.

equally. Although the level of the intron-containing precursor is much greater in *ac20* than in wild-type cells, this transcript is not nearly as abundant as the spliced 7S-3S-23Sγ-23Sδ RNA, which also overaccumulates. In addition, the fact that deletion of the intron from *ac20* did not significantly alleviate the mutant phenotype also argues for the primary defect in *ac20* occurring after splicing and involving ITS processing. It should be pointed out, however, that this does not mean that there is no role for the *ac20* gene product in *Cr.LSU* splicing, only that the reduced splicing efficiency is not primarily responsible for the mutant phenotype.

The composition of the LSU RNA precursors in *ac20* suggests that processing of ITS-1 is the most affected step. Nearly all of the accumulated precursors retain ITS-1, including two novel RNAs (7S-3S and 7S-3S-23Sγ species) that were not seen in wild-type cells (Figure 2); these RNAs do not accumulate in the wild type presumably because of rapid processing of ITS-1 and ITS-2. The disproportionate increase in *ac20* of the 7S-3S-23Sγ-23Sδ RNA, however, more so than the other precursors, suggests that processing of ITS-2 and ITS-3 is also affected, although this result might represent an indirect effect of reduced ITS-1 processing (i.e., the removal of ITS-2 and ITS-3 is inefficient if ITS-1 has not been removed). In either case, these data suggest that ITS processing is important for chloroplast ribosome formation and/or function. Finally, the internal cleavage of 23Sγ RNA also seems to be reduced in *ac20*, although, as discussed below, this cleavage event may not represent a true processing step.

What kind of function does the *ac20* gene product provide? The fact that LSU precursors were detected in wild-type cells suggests that these steps occur after transcription, presumably in ribonucleoproteins. Considering this, and other evidence, the *ac20* gene product could function as an RNA "chaperone" (Herschlag, 1995), promoting correct folding of LSU rRNA but not being required for ribosome function per se. The *E. coli* ribosomal protein S12 has been shown to exhibit RNA chaperone-like activity toward group I introns from the T4 phage (Coetzee et al., 1994). Interestingly, S12 did not bind strongly to the intron core but rather demonstrated preference for the exon sequences. Thus, by analogy, the *ac20* gene product could stimulate splicing of the *Cr.LSU* intron by promoting proper folding of the LSU exons. Of course, in this scenario, ITS processing would also be strongly dependent on the correct folding of LSU pre-RNA (the stems that are cleaved to release the ITSs might not be accessible otherwise).

Alternatively, the *ac20* gene product could encode a ribonuclease responsible for removal of the ITSs, and the minor reduction in splicing efficiency is a secondary effect. Burgin et al. (1990) showed that processing of the ITS in the 23S rRNA of the bacterium *Salmonella typhimurium* could be catalyzed by RNase III from *E. coli.* However, *ac20* is unlikely to be an RNase III–deficient mutant, because this enzyme is also involved in liberating pre-16S and pre-23S from the primary transcript (Gegenheimer and Apirion, 1981).

Cleavage of 23Sγ

These experiments also revealed a previously unknown, in vivo cleavage of 23Sγ RNA (Figures 2 and 3) that is unusual for two reasons. First, unlike the ITSs, where the cleavage sites abut nonconserved nucleotides (cf. Turmel et al., 1991), this site (A505 in the nomenclature for *E. coli* 23S rRNA) maps to a highly conserved region of domain I (Gutell et al., 1990). Thus, this processing event is unlikely to be a new ITS. Second, only ~35% of the 23Sγ RNA is cleaved even in wild-type cells, thus raising the question of whether there is any physiological relevance for this cleavage.

A recent report of site-specific cleavage of *E. coli* rRNA in vitro with lead (Winter et al., 1997) may provide a clue to the meaning of 23Sγ cleavage. Site-specific cleavage with lead ions can be used to identify metal binding sites in RNA (reviewed in Pan et al., 1993). Winter et al. (1997) reported that Pb^{2+} catalyzed cleavage of *E. coli* rRNA at three major sites, one of which they mapped to A505 in 23S. The authors also observed that the lead-cleaved ribosomes were still functional in in vitro assays, indicating that cleavage at A505 did not negatively affect ribosome structure. Thus, we suggest that the cleavage of Chlamydomonas 23Sγ RNA could represent a side reaction generated by a tightly bound metal ion. The fact that only 35 to 40% of the 23Sγ RNA in Chlamydomonas is cleaved might reflect the relative inefficiency of metal-catalyzed cleavage in vivo; the principal divalent metal in vivo is Mg^{2+}, which is not nearly as active as Pb^{2+} in promoting RNA cleavage (Pan et al., 1993).

Role of the *Cr.LSU* Intron

Deletion of the *Cr.LSU* intron from the chloroplast genome (Figure 5) had no observable effect on the growth of wild-type cells. Previously, all four group I introns in the *psbA* gene were shown to be dispensable (Johanningmeier and Heiss, 1993). Thus, none of the five known group I introns in Chlamydomonas encode necessary cellular functions outside of intron-related activities. Likewise, all of the group I introns were removed from the yeast mitochondrial genome without detriment (Séraphin et al., 1987). Thus, it is becoming increasingly evident that the majority of group I introns have little or no essential role in the normal functioning of their cellular hosts. A notable exception, however, is the *LSU* intron of *Neurospora crassa*, which may encode the ribosomal protein S5 (Burke and RajBhandary, 1982).

Although the *Cr.LSU* intron can be deleted from the genome without obvious detriment, in wild-type cells it must be spliced from the precursor RNA to form functional ribosomes. This was indicated by the fact that only heteroplasmic transformants were obtained after transformation with *rrnL* genes containing mutations in the J4/5, P6, or P7 regions of the intron core. The *Cr.LSU* intron is located in a region of 23S rRNA that has been implicated in amino–acyl tRNA binding (Noller, 1991). Thus, the presence of the intron probably interferes with the proper folding of this region.

It was shown, however, that it is possible to obtain homoplasmic transformants with substitutions in the P4 helix of the intron (Figure 6). These strains had reduced levels of mature LSU rRNAs, and they were light and cold sensitive. The light sensitivity is presumably due to a decrease in certain chloroplast translation products (Harris, 1989), whereas cold sensitivity is often a trait of ribosome mutants (Harris et al., 1987).

The P4 mutants contained high levels of unspliced LSU RNA characteristic of a splicing deficiency. These strains also contained novel pre-RNAs that had been processed at either ITS-2 or ITS-3 but retained the intron. This latter finding indicated that ITS processing can occur on unspliced pre-RNA. The ITS processing was inefficient, however, because these mutants also had increased levels of the unprocessed pre-RNA (7S-3S-23Sγ-23Sδ-intron RNA in Figure 6). The decrease in ITS processing in the splicing-deficient mutants seemed to affect all three ITSs about equally, as there was no evidence of a preferential affect on one ITS over the others. Finally, the ability to create splicing-defective intron mutants is significant, because it may allow us to isolate second-site suppressor mutants.

The Pathway for LSU rRNA Processing in Wild-Type Chlamydomonas

Although our analysis is principally one of steady state RNA levels, this approach was used previously to infer the temporal maturation of precursor RNAs (e.g., Kister et al., 1983; Keus et al., 1984). Unlike LSU, precursors of neither 16S nor 5S were clearly detected, suggesting that processing of the LSU pre-RNA is limiting, at least in terms of rRNA maturation.

The proposed pathway for maturation of the 3.8-kb LSU pre-RNA in wild-type Chlamydomonas is diagrammed in Figure 7. The first step is splicing of the group I intron. Because the intron is located downstream from the ITSs, LSU processing is not strictly 5′ to 3′, which contrasts with pre-rRNA maturation in *E. coli* (Gegenheimer and Apirion, 1981). However, this is similar to cytoplasmic LSU rRNA processing in the eukaryote *Tetrahymena thermophilum*, in which the self-splicing group I intron is removed first (Kister et al., 1983).

As shown in Figure 7, splicing is followed by processing of the first two ITSs. These appear to be removed simultaneously. Alternatively, removal of ITS-2 occurs very rapidly once ITS-1 is removed. Either mechanism would explain the absence of a 3S-23Sγ-23Sδ RNA in wild-type cells, which would have been detected on the RNA gel blots of Figure 2A if it had accumulated to any significant extent.

In the final step, processing of ITS-3 separates the remainder of LSU into the 23Sγ and 23Sδ RNAs. The internal cleavage of 23Sγ was not included in Figure 7, because it may not be a bona fide processing step (see discussion above).

An important question is whether the apparent order of LSU processing in the wild type is required or if it mainly reflects the relative rates of different steps. The analysis of *ac20* and the intron mutants supports the latter conclusion: the composition of the LSU transcripts in *ac20* indicated that processing of the ITSs can occur independently of each other, whereas the RNA profile of the intron mutants indicated that splicing is not absolutely required for processing of ITS-2 and ITS-3. Currently, we cannot rule out the possibility that splicing might be required for processing of ITS-1, although this seems unlikely given that the other steps are not absolutely coupled. On the other hand, these data do indicate that for at least some steps, the order affects the efficiency. For example, processing of the ITSs was slowed by the presence of the intron (Figure 6). Thus, the highly efficient processing of pre-LSU rRNA in the wild type is dependent in part on maintaining the relative rates of certain steps.

METHODS

Strains and Culture Conditions

The wild-type strain 2137 mt(+) and the chloroplast ribosome-deficient mutant *ac20* (CC-34) were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC, c/o Lib Harris). These and the other mutant strains constructed in this study were grown in Tris–acetate–phosphate (TAP) medium (Harris, 1989) at 23°C. Unless stated otherwise, the light intensity was ~2000 lux for the light-insensitive strains and ~150 lux for the light-sensitive mutants. Cells were harvested for analysis during the exponential phase

of growth (1 to 2 × 10^6 cells per mL). TAP was supplemented with agar (20 g/L) to make solid medium. Minimal medium was prepared using the recipe for TAP medium, except that the pH was adjusted with hydrochloric acid instead of acetic acid.

In Vitro Mutagenesis and Construction of Plasmids for Transformation

A 23S gene containing an erythromycin resistance marker (Harris et al., 1989) and lacking most of the intron open reading frame (ORF; Thompson and Herrin, 1991) was used for site-directed mutagenesis of the intron. For the mutagenesis of specific nucleotides, the BamHI-HindIII fragment from plasmid p194-1 (Thompson and Herrin, 1991) was recloned into the pTZ18U vector to create pTZ23S.2er. Subsequently, nucleotide substitutions were introduced into the P4, J4/5, P6, and P7 regions of the intron (see Thompson and Herrin, 1991) by using the Muta-Gene Phagemid in vitro mutagenesis kit (Bio-Rad) and following the manufacturer's protocols. The mutagenic oligodeoxynucleotides were as follows: 5′-d(TGGCAACGC-**(R)**GAGGGAAGAC)-3′ (**[R]** is a purine), which was used to change C-126 of the intron (in P4) to an A residue and a G residue; 5′-d(TATCGGTGA**(C)**ACCTTCCTA)-3′ (**[C]** is the point mutation), which was used to change A-59 of the intron (in J4/5) to a C residue; 5′-d(AACGCCGA**(C/A)**GAAGACCAT)-3′, which was used to change G-130 of the intron (in P6) to a C residue and an A residue; and 5′-d(GAAGACATA**(C/A)**TCCATGCCT)-3′, which was used to change G-820 of the intron (in P7) to a C residue and an A residue. The mutations were verified by sequencing the cloned plasmids with oligodeoxynucleotide 8 (Figure 1). Subsequently, the BamHI fragment from plasmid p183, which contains the *spr-u-1-6-2* marker (Harris et al., 1989), was ligated into the BamHI site of the plasmids to create the mutant intron plasmids used for chloroplast transformation (Figure 4A).

Plasmid pGEM23S.int$^-$, which was used to delete the 23S intron in vivo (see Figure 4B), was created in two steps. Initially, the HindIII fragment of p322 (Newman et al., 1992) was cloned into the HindIII site of pGEM23S.E (Thompson et al., 1992), which simultaneously removed the erythromycin resistance marker and added resistance to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU); (which was provided by exon 5 of a DCMU-resistant *psbA* gene [Erickson et al., 1984]). Subsequently, the BamHI fragment of pSR1 was cloned into the flanking BamHI site, thereby adding streptomycin resistance, which was encoded in the 16S gene (Harris et al., 1989). Plasmid pSR1 had been created previously by combining, in the pGEM3Zf(+) vector (Promega), the BamHI-ScaI fragment of pBC4 (Herrin et al., 1990) with the BamHI-ScaI fragment of p183-1 (Newman et al., 1990).

Chloroplast Transformation

A biolistic particle gun (model PDS-1000 He; Bio-Rad) was used for chloroplast transformation. Plasmid DNA, purified by standard methods and linearized by digestion with the appropriate restriction enzyme (PstI for pGEM23S.int$^-$ and PvuI for the mutant intron plasmids), was precipitated onto tungsten microprojectiles (M-17; Bio-Rad), following the manufacturer's protocols. The manufacturer's instructions for transformation were followed using the default settings, with the exception of the following parameters: the helium gas pressure (i.e., rupture disc) was 900 p.s.i., the vacuum was 28 inches of Hg, and the target distance was 6 cm. Cultures to be transformed were grown in continuous bright light (see above) to a density of 1 to 2 × 10^6 cells per mL in TAP medium. The cells were harvested by centrifugation at ~2000*g* and resuspended to a density of 5 × 10^6 cells per mL in TAP medium. This suspension (0.8 mL) was mixed with 0.8 mL of molten, 0.5% TAP agar at 42°C, and plated onto the center of two TAP agar plates, keeping the mixture in a circle of ~5 cm in diameter. This was allowed to set and dry on a leveling table until the surface became matte.

After transformation, the plates were placed in low light (~150 lux) at 23°C overnight. The next day, cells were scraped off and replated onto TAP agar plates with the primary selective antibiotic: spectinomycin (400 μg/mL) for the mutant intron plasmids or streptomycin (400 μg/mL) for pGEM23S.int$^-$. After 10 days of growth under strong light (~2000 lux) for the pGEM23S.int$^-$ plasmid or dim light (~150 lux) for the mutant intron plasmids, colonies were picked and screened for growth (under the same illumination) on the secondary, selective inhibitor. TAP plates with erythromycin (200 μg/mL) were used for the mutant intron plasmids, and minimal plates with DCMU (3 μM) were used for pGEM23S.int$^-$.

Oligodeoxynucleotide Probes

The sequences of the oligodeoxynucleotides indicated in Figure 1 are as follows, in ascending order: 5′-ATATTTTGTATTCATAAGATG-3′ (8), 5′-ACCTATATAACGGCTTGTCT-3′ (14), 5′-TCGCTCAACGGATAA-AAGTT-3′ (15), 5′-GGTGCAGGATTCAGCTGCTTCG-3′ (71), 5′-GCC-TAGGTCTCCACCGTACGCC-3′ (72), 5′-CTATTTCACTCCCCAACA-GGGG-3′ (73), 5′-CCCCTTCCCCTTCGGCGTCC-3′ (74), and 5′-CTC-GCT TTCGCTTGGGCTCCGG-3′ (76).

Nucleic Acids Isolation, Polymerase Chain Reaction, and Sequencing

Nucleic acids were isolated as described previously (Herrin and Schmidt, 1988). Polymerase chain reaction (PCR) was performed using Taq DNA polymerase with the supplied buffer (Perkin-Elmer Cetus), as suggested by the manufacturer. The reaction mixtures (50 μL) contained 4 μg of total nucleic acid, 100 pmol of each primer (oligodeoxynucleotides 14 and 15), 200 μM of each deoxynucleotide triphosphate, and 2.5 units of polymerase. Amplification was achieved by 30 cycles of 94°C (1 min), 46°C (2 min), and 72°C (2 min). The products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

PCR products were purified by agarose gel electrophoresis by using centrifugal filters (Millipore, Bedfore, MA), according to the manufacturer's recommendations. The DNAs were sequenced by using Sequenase version 2.0 (U.S. Biochemical Corp.) and following the manufacturer's suggestions, except that the template was denatured with heat rather than alkali.

Blotting and Hybridization

Alkaline–DNA gel blotting onto nylon membranes (Zeta probe) was performed according to the manufacturer's instructions (Bio-Rad). For RNA gel blotting, total cellular RNA was separated by electrophoresis in 1.4% agarose/2% formaldehyde gels and blotted, as described previously (Herrin et al., 1990). The blots were reversibly stained with methylene blue to verify equal loading and transfer (Herrin and Schmidt, 1988). Cloned DNA probes were labeled with α–^{32}P-

dCTP (~3000 Ci/mmol) by the random priming method (Feinberg and Vogelstein, 1983). Hybridizations were performed as described previously (Herrin et al., 1990); final washes were at 50°C in 0.2 × SSPE (1 × SSPE is 0.15 M NaCl, 50 mM NaH_2PO_4, pH 7.4, and 5 mM EDTA) and 0.5% SDS.

Oligodeoxynucleotide probes were 5′ end labeled with T4 polynucleotide kinase and γ-^{32}P-ATP (~5000 Ci/mmol) to a specific activity of ~10^7 cpm/μg. Hybridization with the oligodeoxynucleotides was performed in 6 × SSPE, 1% SDS, 10 × Denhardt's solution (1 × Denhardt's solution is 0.2% BSA, 0.2% Ficoll 400, and 0.2% PVP) at 42°C; the probe concentration was ~10 ng/mL. After hybridization, the blots were washed at a temperature ~5°C below the melting temperature in 6 × SSPE and 0.2% SDS three times for 3 min and then three times for 6 min. Exposure of the blots to x-ray film was at −70°C with an intensifying screen.

Primer Extension

Primer extension analysis was performed using total cellular RNA and oligodeoxynucleotides 73 and 76 (5′ end labeled) in the presence of 75 μg/mL actinomycin D (Boorstein and Craig, 1989). The published procedure was modified by performing the annealing step at 42°C for 30 min, and the RNA was not removed before electrophoresis of the extension products on denaturing polyacrylamide gels.

ACKNOWLEDGMENTS

This research was supported by grants from the U.S. Department of Agriculture (National Research Initiative Competitive Grants Program No. 96-35301-3420), the Robert A. Welch Foundation (No. F-1164), and the Texas Advanced Technology Program (No. ATP-003658-326).

Received January 23, 1998; accepted May 6, 1998.

REFERENCES

Barkan, A. (1993). Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. Plant Cell **5,** 389–402.

Boorstein, W.R., and Craig, F.A. (1989). Primer extension analysis of RNA. Methods Enzymol. **180,** 347–369.

Boynton, J., Gillham, N.W., and Burkholder, B. (1970). Mutations altering chloroplast ribosome phenotype in *Chlamydomonas.* II. A new Mendelian mutation. Proc. Natl. Acad. Sci. USA **67,** 1505–1512.

Burgin, A.B., Parodos, K., Lane, D.J., and Pace, N.R. (1990). The excision of intervening sequences from *Salmonella* 23S ribosomal RNA. Cell **60,** 405–414.

Burke, J.M., and RajBhandary, U.L. (1982). Intron within the large rRNA gene of *N. crassa* mitochondria: A long open reading frame and a consensus sequence possibly important in splicing. Cell **31,** 509–520.

Coetzee, T., Herschlag, D., and Belfort, M. (1994). *E. coli* proteins, including ribosomal protein S12, facilitate in vitro splicing of phage T4 introns by acting as RNA chaperones. Genes Dev. **8,** 1575–1588.

Dron, M., Rahire, M., and Rochaix, J.-D. (1982). Sequence of the chloroplast 16S rRNA gene and its surrounding regions of *Chlamydomonas reinhardtii.* Nucleic Acids Res. **10,** 7609–7619.

Dujon, B. (1989). Group I introns as mobile genetic elements: Facts and mechanistic speculations—A review. Gene **82,** 91–114.

Durrenberger, F., and Rochaix, J.-D. (1991). Chloroplast ribosomal intron of *Chlamydomonas reinhardtii*: In vitro self-splicing, DNA endonuclease activity and in vivo mobility. EMBO J. **10,** 3495–3501.

Durrenberger, F., Thompson, A.J., Herrin, D.L., and Rochaix, J.-D. (1996). Double strand break-induced recombination in chloroplasts of *Chlamydomonas reinhardtii.* Nucleic Acids Res. **24,** 3323–3331.

Erickson, J.M, Rahire, M., Bennoun, P., Delepelaire, P., Diner, B., and Rochaix, J-D. (1984). Herbicide resistance in *Chlamydomonas reinhardtii* results from a mutation in the chloroplast gene for the 32-kilodalton protein of photosystem II. Proc. Natl. Acad. Sci. USA **81,** 3617–3621.

Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **257,** 8569–8572.

Gegenheimer, P., and Apirion, D. (1981). Processing of procaryotic ribonucleic acid. Microbiol. Rev. **45,** 502–541.

Gutell, R.R., Schnare, M.N., and Gray, M.W. (1990). A compilation of large subunit (23S-like) ribosomal RNA sequences presented in a secondary structure format. Nucleic Acids Res. **18,** 2319–2330.

Harris, E.H. (1989). The *Chlamydomonas* Sourcebook. (San Diego, CA: Academic Press).

Harris, E.H., Boynton, J.E., and Gillham, N.W. (1974). Chloroplast ribosome biogenesis in *Chlamydomonas.* Selection and characterization of mutants blocked in ribosome formation. J. Cell Biol. **63,** 160–179.

Harris, E.H., Boynton, J.E., and Gillham, N.W. (1987). Interaction of nuclear and chloroplast mutations in biogenesis of chloroplast ribosomes in *Chlamydomonas.* In Algal Development: Molecular and Cellular Aspects, W. Wiessner, D.G. Robinson, and R.C. Starr, eds (Berlin: Springer-Verlag), pp. 142–149.

Harris, E.H., Burkhart, B.D., Gillham, N.W., and Boynton, J.E. (1989). Antibiotic resistance mutations in the chloroplast 16S and 23S rRNA genes of *Chlamydomonas reinhardtii*: Correlation of genetic and physical maps of the chloroplast genome. Genetics **123,** 281–293.

Harris, E.H., Boynton, J.E., and Gillham, N.W. (1994). Chloroplast ribosomes and protein synthesis. Microbiol. Rev. **58,** 700–754.

Hartley, M.R. (1979). The synthesis and origin of chloroplast low-molecular-weight ribosomal ribonucleic acid in spinach. Eur. J. Biochem. **96,** 311–320.

Herrin, D.L., and Schmidt, G.W. (1988). Rapid reversible staining of northern blots prior to hybridization. BioTechniques **3,** 196–198.

Herrin, D.L., Chen, Y.-F., and Schmidt, G.W. (1990). RNA splicing in *Chlamydomonas* chloroplasts. Self-splicing of 23S preRNA. J. Biol. Chem. **265,** 21134–21140.

Herschlag, D. (1995). RNA chaperones and the RNA folding problem. J. Biol. Chem. **270,** 20871–20874.

Hill, J., McGraw, P., and Tzagoloff, A. (1985). A mutation in yeast mitochondrial DNA results in a precise excision of the terminal intron of the cytochrome *b* gene. J. Biol. Chem. **260,** 3235–3238.

Johanningmeier, U., and Heiss, S. (1993). Construction of a *Chlamydomonas reinhardtii* mutant with an intronless *psbA* gene. Plant Mol. Biol. **22,** 91–99.

Kapoor, M., Nagai, T., Wakasugi, T., Yoshinaga, K., and Sugiura, M. (1997). Organization of chloroplast ribosomal RNA genes and in vitro self-splicing activity of the large subunit rRNA intron from the green alga *Chlorella vulgaris* C-27. Curr. Genet. **31,** 503–510.

Keus, R.J.A., Dekker, A.F., Kreuk, K.C.J., and Groot, G.S.P. (1984). Transcription of ribosomal DNA in chloroplasts of *Spirodela oligorhiza.* Curr. Genet. **9,** 91–97.

Kister, K.P., Müller, B., and Eckert, W.A. (1983). Complex endonucleolytic cleavage pattern during early events in the processing of pre-rRNA in the lower eukaryote, *Tetrahymena thermophila.* Nucleic Acids Res. **11,** 3487–3502.

Kössel, H., Natt, E., Strittmatter, G., Fritzsche, E., Gozdzicka-Jozefiak, A., and Przybyl, D. (1985). Structure and expression of rRNA operons from plastids of higher plants. In Molecular Form and Function of the Plant Genome, L. Van Vloten-Doting, G.S.P. Groot, and T.C. Hall, eds (New York: Plenum Press), pp. 183–198.

Leaver, C.J., and Ingle, J. (1971). The molecular integrity of chloroplast ribosomal ribonucleic acid. Biochem. J. **123,** 235–243.

Lemieux, C., Boulanger, J., Otis, C., and Turmel, M. (1989). Nucleotide sequence of the chloroplast large subunit rRNA gene from *Chlamydomonas reinhardtii.* Nucleic Acids Res. **17,** 7997.

Liu, X.-Q., Gillham, N.W., and Boynton, J.E. (1988). Chloroplast ribosomal protein L-18 in *Chlamydomonas reinhardtii* is processed during ribosome assembly. Mol. Gen. Genet. **214,** 588–591.

Michel, F., and Westhof, E. (1990). Modelling of the three-dimensional architecture of group I catalytic introns based on comparative sequence analysis. J. Mol. Biol. **216,** 585–610.

Myers, A.M., Harris, E.H., Gillham, N.W., and Boynton, J.E. (1984). Mutations in a nuclear gene of *Chlamydomonas* cause the loss of two chloroplast ribosomal proteins, one synthesized in the chloroplast and the other in the cytoplasm. Curr. Genet. **8,** 369–378.

Newman, S.M., Boynton, J.E., Gillham, N.W., Randolph-Anderson, B.L., Johnson, A.M., and Harris, E.H. (1990). Transformation of chloroplast ribosomal RNA genes in *Chlamydomonas.* Molecular and genetic characterization of integration events. Genetics **126,** 875–888.

Newman, S.M., Harris, E.H., Johnson, A.M., Boynton, J.E., and Gillham, N.W. (1992). Nonrandom distribution of chloroplast recombination events in *Chlamydomonas reinhardtii*: Evidence for a hotspot and an adjacent cold region. Genetics **132,** 413–429.

Noller, H. (1991). Ribosomal RNA and translation. Annu. Rev. Biochem. **60,** 191–227.

Pan, T., Long, D.M., and Uhlenbeck, O.C. (1993). Divalent metal ions in RNA folding and catalysis. In The RNA World, R.F. Gesteland and J.F. Atkins, eds (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 271–302.

Rochaix, J.-D., and Darlix, J.-L. (1982). Composite structure of the chloroplast 23S ribosomal RNA genes of *Chlamydomonas reinhardtii.* Evolutionary and functional implications. J. Mol. Biol. **159,** 383–395.

Rochaix, J.-D., and Malnoe, P. (1978). Anatomy of the chloroplast ribosomal DNA of *Chlamydomonas reinhardtii.* Cell **15,** 661–670.

Rochaix, J.-D., Rahire, M., and Michel, F. (1985). The chloroplast ribosomal intron of *Chlamydomonas reinhardtii* codes for a polypeptide related to mitochondrial maturases. Nucleic Acids Res. **13,** 975–984.

Schmidt, R.J., Gillham, N.W., and Boynton, J.E. (1985). Processing of the precursor to a chloroplast ribosomal protein made in the cytosol occurs in two steps, one of which depends on a protein made in the chloroplast. Mol. Cell. Biol. **5,** 1093–1099.

Schneider, M., and Rochaix, J.-D. (1986). Sequence organization of the chloroplast ribosomal spacer of *Chlamydomonas reinhardtii*: Uninterrupted $tRNA^{Ile}$ and $tRNA^{Ala}$ genes and extensive secondary structure. Plant Mol. Biol. **6,** 265–270.

Schneider, M., Darlix, J.L., Erickson, J., and Rochaix, J.-D. (1985). Sequence organization of repetitive elements in the flanking regions of the chloroplast ribosomal unit of *Chlamydomonas reinhardtii.* Nucleic Acids Res. **13,** 8531–8541.

Séraphin, B., Boulet, A., Simon, M., and Faye, G. (1987). Construction of a yeast strain devoid of mitochondrial introns and its use to screen nuclear genes involved in mitochondrial splicing. Proc. Natl. Acad. Sci. USA **84,** 6810–6814.

Shepherd, H.S., Boynton, J.E., and Gillham, N.W. (1979). Mutations in nine chloroplast loci of *Chlamydomonas* affecting different photosynthetic functions. Proc. Natl. Acad. Sci. USA **76,** 1353–1357.

Thompson, A.J., and Herrin, D.L. (1991). In vitro self-splicing reactions of the chloroplast group I intron *Cr.LSU* from *Chlamydomonas reinhardtii* and in vivo manipulation via gene-replacement. Nucleic Acids Res. **23,** 6611–6618.

Thompson, A.J., Yuan, X., Kudlicki, W., and Herrin, D.L. (1992). Cleavage and recognition pattern of a double-strand–specific endonuclease (I-*Cre*I) encoded by the chloroplast 23S rRNA of *Chlamydomonas reinhardtii.* Gene **119,** 247–251.

Turmel, M., Boulanger, J., Schnare, M.N., Gray, M.W., and Lemieux, C. (1991). Six group I introns and three internal transcribed spacers in the chloroplast large subunit ribosomal RNA gene of the green alga *Chlamydomonas eugametos.* J. Mol. Biol. **218,** 292–311.

Turmel, M., Gutell, R.R., Mercier, J.P., Otis, C., and Lemieux, C. (1993). Analysis of the chloroplast large subunit ribosomal RNA gene from 17 *Chlamydomonas* taxa: Three internal transcribed spacers and 12 group I intron insertion sites. J. Mol. Biol. **232,** 446–467.

Winter, D., Polacek, N., Halama, I., Streicher, B., and Barta, A. (1997). Lead-catalyzed cleavage of ribosomal RNAs. Nucleic Acids Res. **25,** 1817–1824.